THE PRINCIPLE OF IMMERSION MICROREFRACTOMETRY FOR THE DIRECT DETERMINATION OF LIVING AND DEAD BACTERIAL CELLS

Translation No. 1708

February 1965

U. S. ARMY
BIOLOGICAL LABORATORIES
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/Following is the translation of an article by B. A. Fikhman, L. A. Tarasevich State Control Institute for Medicinal Biological Preparations, appearing in the Russian-language periodical Doklady Akademii Nauk SSSR (Reports of the USSR Academy of Sciences) 1959, Vol 124, No. 5, pages 1141-1143. It was submitted on 6 Oct 1958 and presented by Academy Member V. N. Shaposhnikovyy on 16 Oct 1958. Translation performed by Sp/7 Charles T. Ostertag Jr./

The determination of the number of living and dead cells and their quantitative relationship in microbial populations or suspensions has great significance for the various fields of practical and experimental microbiology. In spite of the large number of methods proposed (cultural, morphological, chemical, cytochemical), up until the present time microbiological technique does not have available a sufficiently accurate, objective and simple method for the direct differentiated calculation of living and dead bacterial cells. This is explained by the fact that the methods used at the present time, regardless of the principle they are based on, do not make it possible directly, without additional effects and with a sufficient authenticity, to ascertain the setting in of death of the bacterial cell. In connection with this, attention is merited by the phenomenon of the irreversible changing of the optical properties (increase of optical density and light refractive index) of the cells as a result of their death. A sufficiently sharp exposure and quantitative evaluation of the stated phenomenon became possible only after the introduction of microscopic investigation by the phase-contrast method. An important improvement of the method was the use of special immersion media (1--3, 5). The use of exact concentration of albumin solutions made it possible (4) to develop a microrefractometric method for determining the concentration of dry substances and water in living cells.

As is known, the phase-contrast image reflects the degree of optical density and the general correlations of the light refractive index of the object connected with it. Along with this, the intensity of the image of an object, immersed in this or that medium, depends not only on the difference of the phase generated by it, but on the gradient of this difference. The stated circumstance is the reason that during ordinary phase-contrast microscopy the human eye, incapble of quantitatively evaluating the intensity of the image, does not always realize the sensitivity of the method. Special tests, conducted with the aim of establishing the optimum conditions for exposing differences in light refraction between living and dead cells, showed that the maximum optical differentiation between them is achieved by using immersion media with a light refractive index equal to or slightly exceeding the light refractive index of the protoplasm of the living cells. Under these conditions, the eye easily picks up differences in light refraction between the dead cells and the background of the preparation, which is the immersion medium, and the

living cells which are no different from it based on the light refractive index.

When testing the various immersion media, the best results were obtained with gelatinous gels, which possess an optical isotropism and absence of primary orientation of particles, harmlessness for living cells and optical transparency. Besides this, the elasticity and density of gels ensure the satisfactory fixation and non-movement of the cells. The index of light refraction of gelatinous gel in the most important diapason (from 1.350 to 1.390) is found in linear dependency on the concentration of the gelatin. Our determination of the light refractive index of gelatinous gels of various concentrations with the help of an original model of a polarization interferometer designed by N. M. Melankholin (4), showed the following dependency:

Concentration of gelatin	10	15	20	25	30	35	40
Light refractive index	1.350	1.358	1.366	1.374	1.382	1.390	1.397

These data are close to those obtained by Muller (3).

The selection of the concentration of gel depends on the species of microorganism. Thus, 22% gelatinous gel, based on the light refractive index, is closest to the light refractive index of the protoplasm of B. coli cells. For plague microbes the closest is 23% gelatinous gel, and for staphylococcus -- 27%.

During anoptral microscopy (ordinary phase objectives, possessing a lower resolving capability, give less clearer results) of living and dead cells immersed in gelatinous gel of the appropriate concentration, the following is observed: On the light brown background of the preparation along with the brightly shining cells there are cells -- "vacuums" -- visible, clearly edged with a shining thin rim -- the cell wall. These two types of cells, of which the first correspond to dead, and the second to living cells, are seen very clearly and their optical differentiation is exceedingly simple. In some living cells, especially when investigating young cultures, the so-called conversion of contrast is observed, that is, the protoplast of such cells is of a darker background. This phenomenon, caused by the lower light refractive index of the stated cells in comparison with other living cells of the population, testifies to the high degree of dispersion of colloids of protoplasm and indicates an increased intensity in the life activity of the given cells. Direct observations of the subsequent development of these cells showed that the majority of living (optically "empty") cells display all the symptoms of growth and division. Some of the living cells died during the observations, having transformed into luminous cells. The occurrence of growth or division in the luminous cells was not noted.

Intermediate forms (cells with walls of an unequal thickness and shining sections of protoplasm) apparently represent various degrees of cell degeneration. Direct observation of the subsequent development of such cells showed that only some of them with the least expressed signs of "local" degeneration are capable of division. As regards secondary changes, endured by the dead cells during

the process of autolysis, then depending on the speed of onset, intensity and localization of these processes there is a greater or lesser amount of optically "empty" microsectors in the luminescent cells.

The described optical phenomenon finds an explanation in the fact that with an equality of the light refractive indices of the immersion medium and the protoplasm of a living cell, the latter, when examined in an anoptral microscope, which represents a highly sensitive microrefractometer, disappears optically. During this, the cell wall, the light refractive index of which is considerably higher than the corresponding indices of the immersion medium and the protoplasm, is clearly visible in the form of a light, thin, rim (negative phase-contrast). As regards the dead cells, they, as a result of the sharp increase of the light refractive index caused by the necrobiotic coagulation of colloids of protoplasm, are apparent in the form of shining bodies.

The stated difference in light refraction between living and dead cells becomes sharper yet when the osmotic pressure of the immersion medium is lower than the osmotic pressure of the cells themselves. In this case a sharp lowering of the light refractive index of the living cells is observed due to a certain amount of swelling. Along with this, the light refractive index of the dead cells does not change. The increasing difference caused by this between the light refractive indices of the living and dead cells makes their optical differentiation particularly sharp.

Such sharp changes in the optical properties of a cell after the life processes cease in it are caused primarily by irreversible disturbances of the molecular parameters of protein fractions, setting in as a result of the disorganization of living protoplasm.

The simplicity of the exposure and quantitative evaluation of the described phenomenon opens new possibilities in the study of a number of theoretical and practical problems in the cytology of the microbial cell (bacteriophage, anabiosis, permeability of the cell wall, reactions of the cell to various damaging agents, etc.). The described phenomenon may also be utilized for developing a promising practical method for determining the number of living and dead bacterial cells.

GRAPHIC NOT REPRODUCIBLE

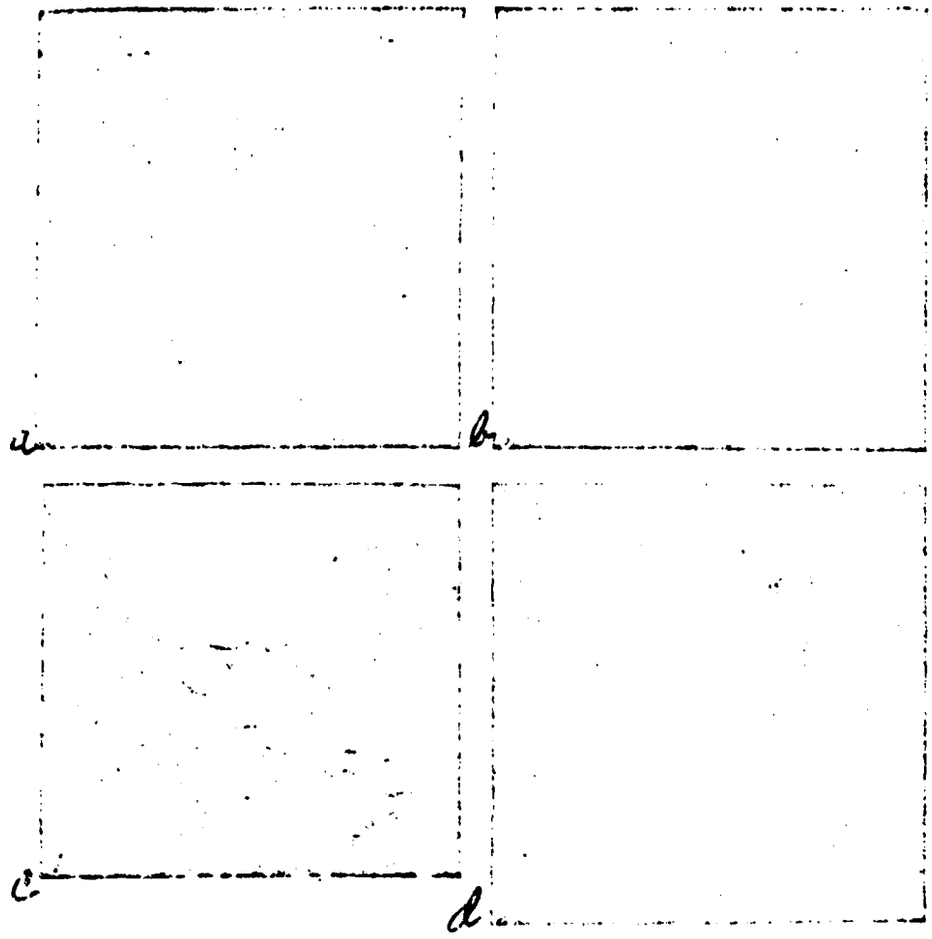

Figure 1. a -- 24 hour culture of B. pestis EV-76 on agar; b -- 48 hour culture of B. Dysent. Flex. 170 on agar; c -- microcolony of S. typhi 1184 on gelatin; d -- 24 hour broth culture of B. Dysent. Sonne 5063. Living and dead cells are visible.